Role of copper during carbon monoxide binding to terminal oxidases

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Abstract Under fully reduced conditions, reassociation kinetics of CO were studied in several terminal oxidases containing copper in their binuclear center. The purified *Paracoccus denitrificans* ba_3-type quinol oxidase was found to recombine with CO monophasically (τ 25–30 ms) like oxidases of the *bo* type from *Escherichia coli*, the caa_3 type from *Bacillus halodurans* FTU, and the *bo* type from *Methylobacillus flagellatum* KT. Oxidase of the aa_3 type from bovine heart recombined with CO monophasically at a higher rate (τ 16–19 ms) than the studied copper-containing bacterial oxidases. After prolonged incubation in the presence of CO, oxidases of the ba_3 and aa_3 types changed their CO-binding properties. The contribution of the slow component was diminished while new fast components arose. Measurement of the metal content in the oxidases indicated that during the incubation, the enzymes lost their copper, the process being accompanied by the appearance of a fast CO recombination rate resembling that of the non-copper oxidases of the *bd* type from *E. coli* and the *bb* type from *Bacillus halodurans* FTU. This points to a role of copper in CO binding by terminal oxidases.

Key words: Bacterial terminal oxidase; Heme-copper binuclear center; Cytochromes ba_3, caa_3, *bo*, *bb*, *bd*; Carbon monoxide binding; Copper; Iron

1. Introduction

Several classifications have been suggested for bacterial oxidases. One of them is based on the type of electron donor, i.e. quinol or cytochrome *c* [1]. On the other hand, largely based on amino acid sequence comparisons, a large superfamily of oxidases can be identified containing a heme-copper binuclear center and translocating protons across the membrane [2]. This superfamily includes both quinol and cytochrome *c* oxidases. Laser flash photolysis of CO-oxidase complexes proves useful as an alternative to identify this enzyme superfamily. According to our data, copper-containing oxidases reassociate with CO upon flash photolysis at a much lower rate than oxidases devoid of the copper cofactor [3–5].

No such data are available for the recently purified novel quinol oxidase of the ba_3 type from *Paracoccus denitrificans* [6]. A homologous cytochrome *c* oxidase of the ba_3 type from *Thermus thermophilus* was reported to have very fast kinetics of CO recombination like the *Escherichia coli bd*-type oxidase [7] whereas another group reported even slower kinetics compared with the bovine heart aa_3-type oxidase [8]. At least four different ba_3-type oxidases have been identified in eubacteria (*P. denitrificans* [6,9], *T. thermophilus* [10], *Broehothrix thermosphacta* [11]) and in the archaebacterium *Natronobacterium pharaonis* [12]. In all cases, the ba_3-type oxidase was shown to contain copper (one atom per molecule in quinol oxidase [6] and an additional Cu_A center in cytochrome *c* oxidase [10]). Interestingly, the ba_3-type oxidase is induced by lowering the O_2 concentration during growth [11,13], which is characteristic of *bd*- [14] and *bb*-type oxidases [15].

The role of copper in the *E. coli bo*-type oxidase was discussed recently. For *E. coli bo*-type oxidase mutant strains defective in putative copper ligands, the rate of CO reassociation upon laser flash increased compared with the wild type enzyme [16]. Moreover, the same enzyme from *E. coli* grown under copper limitation conditions showed about one order higher reassociation rates compared with the enzyme from *E. coli* grown in the presence of copper [17]. However, no direct measurements of copper in oxidases used for the CO reassociation experiments have been done. In this paper, we present a comparative study on CO recombination with a mammalian and several bacterial oxidases. Lowering of copper content in these enzymes was directly accompanied by a rise of fast components in CO recombination kinetics of the bacterial ba_3-type and the mammalian aa_3-type oxidases.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacillus halodurans FTU used in the study is *Bacillus* sp. FTU recently renamed based on additional microbiological characteristics and DNA-DNA hybridization data [18]. The strain was maintained and cultivated as described earlier [15] Two *E. coli* strains, GO102/pFH 101-GO102:F^-; *cyo 123*, *rps L*, *rel A*, *lon 100*, *thi*, *gall*, Δ*cyd::kan*, Str^r, Kan^r; pF 101:pBR 322 (contains no *bo*-type oxidase and overproduces *bd*-type oxidase) and GO103/GR70N, Δ*cyd::kan*, Str^r, Kan^r (contains no *bd*-type oxidase and produces *bo*-type oxidase), were kindly donated to us by Prof. R.B. Gennis. Both strains were grown to late logarithmic phase on LB medium containing 1% NaCl, 1% tryptone and 0.5% yeast extract, pH was adjusted to 7.2 with KOH. *M. flagellatum* KT was cultivated as described earlier [19]. Growth of *P. denitrificans* strain G440 which is deleted in the *fbc* operon and overproduces the ba_3-type quinol oxidase was done according to previously published methods [9].

2.2. Preparation of membranes and enzymes

The caa_3-type oxidase was purified from *B. halodurans* FTU. Membranes were obtained from *B. halodurans* FTU cells as before [15]. The enzyme was extracted by 35 mM octyl glucoside and then fractionated by ammonium sulfate. The fraction obtained as a precipitate at 60–70% ammonium sulfate saturation was fractionated subsequently on a DEAE-Toyo-Pearl (Toyo-Soda, Japan) column and on a Mono Q column (Pharmacia, Sweden). The obtained enzyme contained 16 nmol heme *a* per mg protein. The protein was stored in a medium containing 40 mM MOPS (pH 7.6), 5 mM NaCl, 0.2 mM EDTA, 25 mm octyl glucoside and 25% glycerol in liquid nitrogen. The procedure of isolation of *bb*-type oxidase from *B. halodurans* FTU has been described elsewhere [3]. To obtain membranes from

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Abbreviations: CO, carbon monoxide; Octyl glucoside, *n*-octyl-β-D-glucopyranoside

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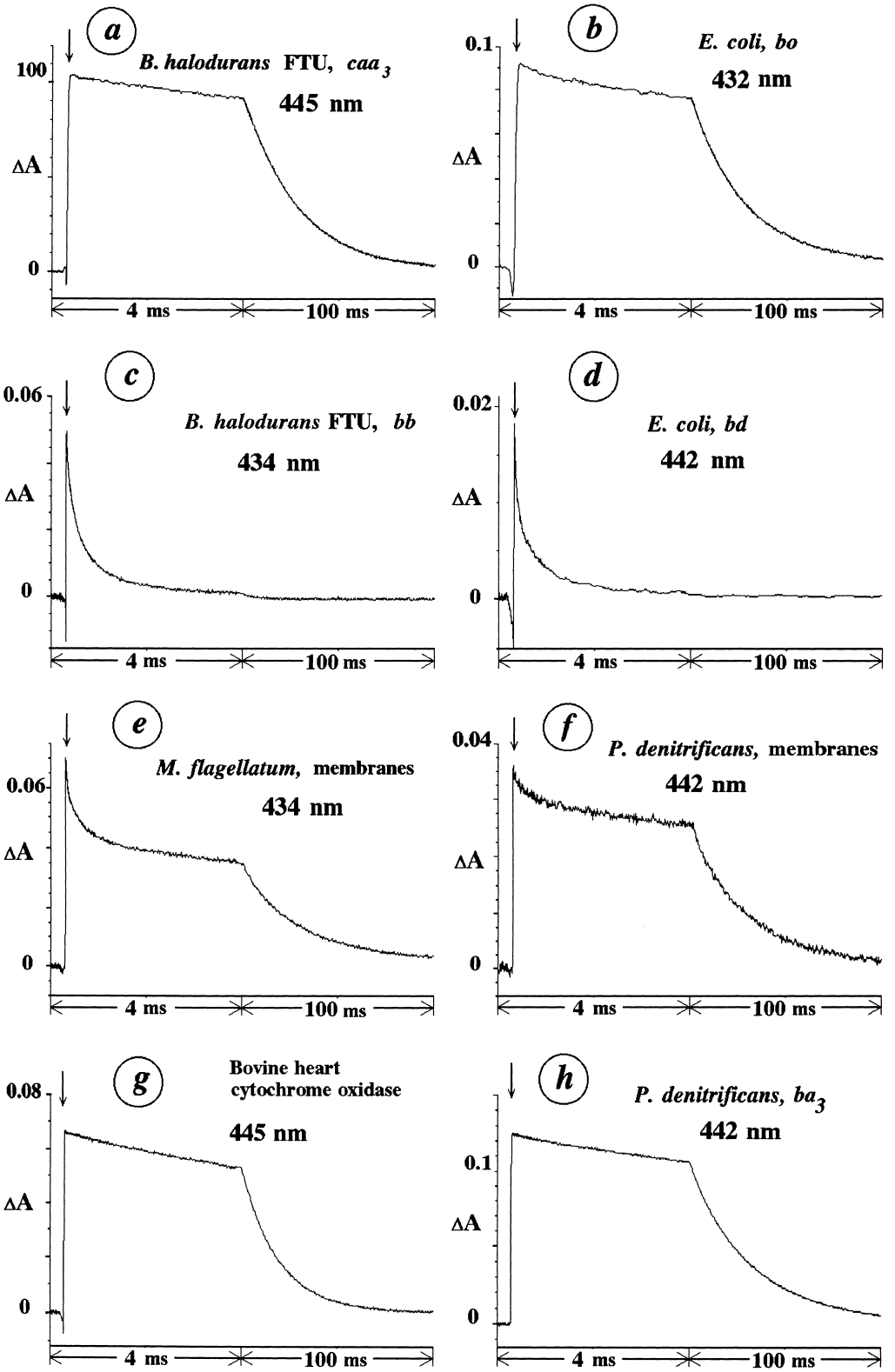

Fig. 1. Kinetics of the CO reassociation with several oxidases under fully reduced conditions upon laser flash photolysis. The samples were flushed with argon for 5 min, reduced by sodium dithionite and gently bubbled with CO for 5 min. Arrow: CO flashed off by the laser. The following enzymes were used in the experiments: (a) *B. halodurans* FTU caa_3-type oxidase, (b) *E. coli* GO103 membranes enriched with *bo*-type oxidase, the *bd*-type oxidase gene being deleted, (c) *B. halodurans* FTU *bb*-type oxidase, (d) *E. coli* GO102 membranes enriched in *bd*-type oxidase, the *bo*-type oxidase gene being deleted, (e) *M. flagellatum* KT membranes, (f) *P. denitrificans* membranes enriched in the ba_3-type oxidase, (g) bovine heart aa_3-type oxidase, (h) purified *P. denitrificans* ba_3-type oxidase. In all cases, the samples were analyzed at wavelengths (indicated in the center of each panel) corresponding to the minimum of their CO difference absorbance spectra in the Soret region.

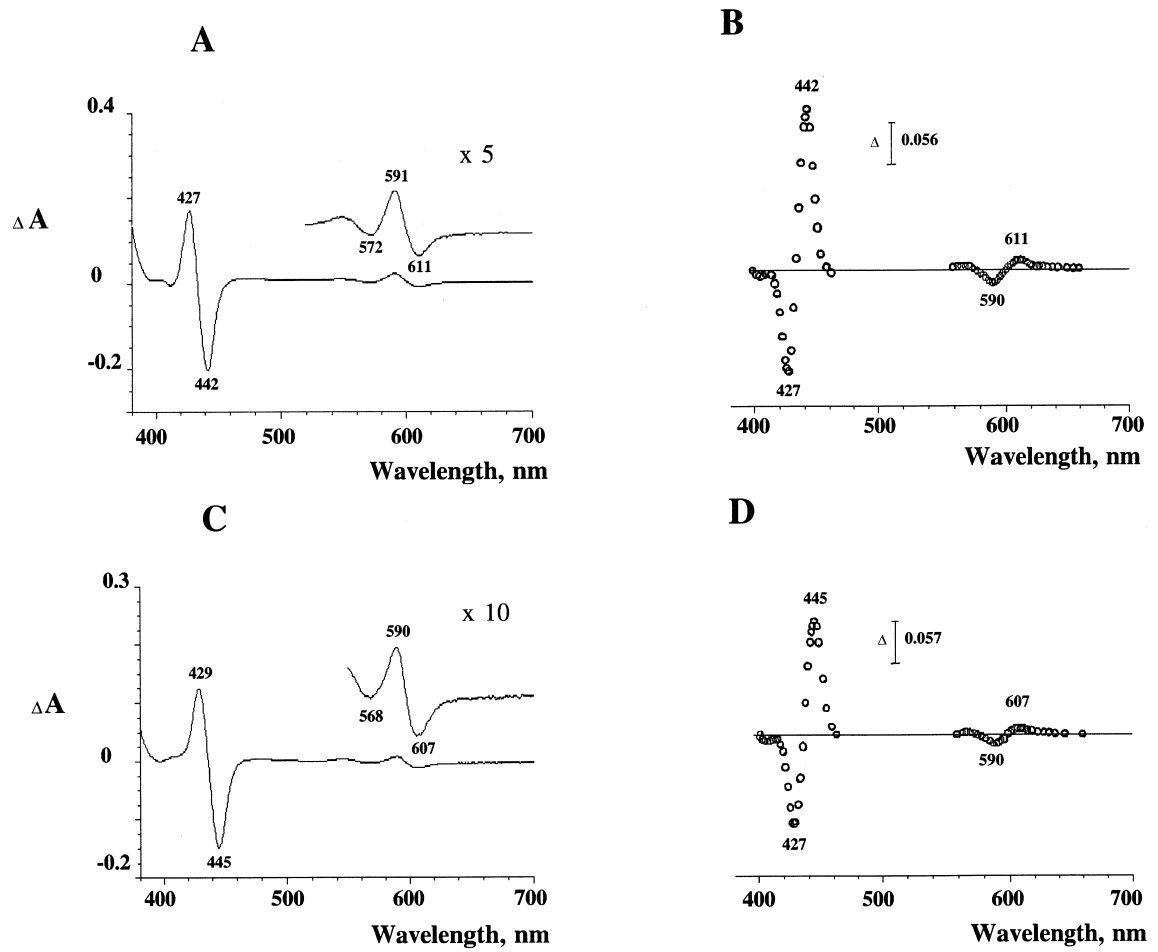

Fig. 2. CO difference absorbance spectra of *P. denitrificans* purified ba_3-type oxidase (A) and bovine heart aa_3-type oxidase (C). For comparison the spectra of laser flash-induced absorbance changes are presented for sample A in panel B, and for sample C in panel D. The samples were treated with argon and CO in the same conditions as in Fig. 1. CO difference absorbance spectra were recorded in 5 min upon bubbling the samples with CO. The spectra of laser flash-induced absorbance changes were recorded for 1 h with the same procedure. Open circles indicate the maxima of absorbance changes at the corresponding wavelengths upon laser flash photolysis of CO oxidase complexes.

E. coli the procedure described by Muntyan et al. was used [4]. Isolation of membranes from *P. denitrificans* strain G440 and preparation of the ba_3-type quinol oxidase were done according to previously reported methods [9]. Membranes from *M. flagellatum* KT were obtained using the procedure described by Muntyan et al. [19]. The aa_3-type oxidase from bovine heart was a gift from Dr. T. Vygodina and contained 9 nmol heme *a* per mg protein. The protein was determined by the Markwell et al. method [20] with BSA as a standard.

2.3. Laser flash photolysis experiments

The experiments were conducted at 20°C using a YG-481 neodymium laser (λ=532 nm; pulse half-width, 15 ns; energy, 70 mJ per flash) (Quantel, France). Details of measurements have been described elsewhere [4]. The enzymes were taken up in a buffer containing 50 mm Tricine-NaOH (pH 8.0), 140 mM KCl, 10 mM NaCl, 0.5 mM EDTA, and 30 mM octyl glucoside. When CO reassociation with enzymes was studied during prolonged incubation periods, a bandpass filter of 0.4 μs time constant was used. Usually about 300–500 kinetic curves obtained as the laser flash-induced optical absorbance changes were stored with 1 s intervals and averaged.

2.4. Metal measurement

Before copper and iron determinations, the enzymes were dialyzed overnight at 4°C against 1000 volumes of buffer containing 10 mM Tris-HCl (pH 7.4) and 10 mM EDTA. Copper and iron were determined by inductively coupled plasma atomic emission spectroscopy [21] using an ICAP 9000 spectrophotometer (Thermo Jarrell Ash, USA).

3. Results

Membranes of *P. denitrificans*, like the purified ba_3-type oxidase, showed a slow rate of CO reassociation upon flash photolysis under fully reduced conditions (Fig. 1f). A small contribution of a faster component in membranes might be due to the presence of *cb*-type nitric oxide reductase which was shown to contain no copper [22] like *B. halodurans* FTU *bb*-type oxidase [5]. Recombination of the ba_3-type oxidase and CO is monophasic (τ 25–30 ms) (Fig. 1h), which is very close to other bacterial oxidases containing a heme-copper binuclear center (Fig. 1a,b). The bovine heart aa_3-type oxidase (Fig. 1g) differs in this respect from all bacterial oxidases showing either slower (Fig. 1a,b) or faster rates of CO recombination (Fig. 1c,d). The kinetic curve of bovine heart oxidase recombination with CO is monophasic (τ 16–19 ms). Analysis of CO recombination curves at a wavelength range of 400–650 nm upon laser flash showed that spectra of the absorbance changes of monophasic components of the ba_3- and aa_3-type oxidases (Fig. 2B,D) fit the CO difference absorbance spectra (Fig. 2A,C). It is well seen that in the cytochrome ba_3, a_3 is the only high-spin heme and heme *b* does not bind CO being low-spin.

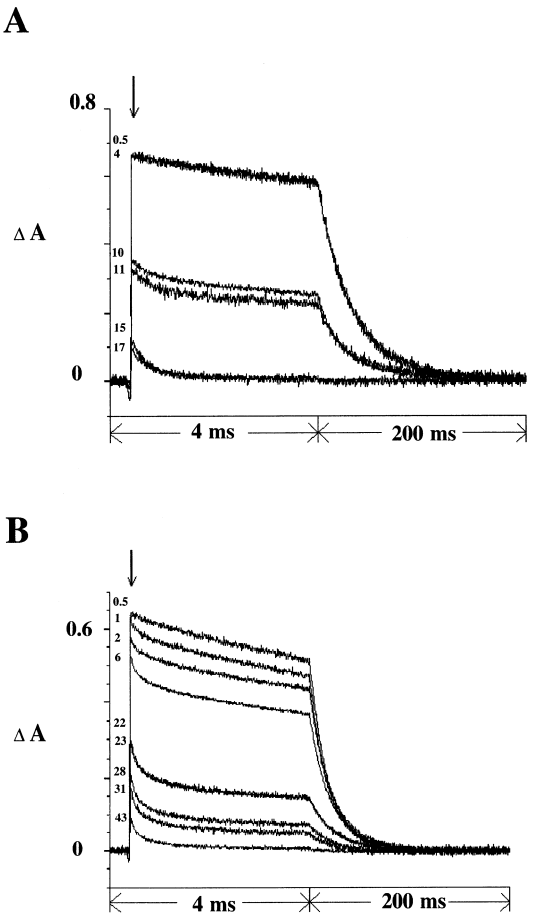

Fig. 3. The time dependence of the CO reassociation kinetics with *P. denitrificans* ba_3-type oxidase at 442 nm (A) and with bovine heart aa_3-type oxidase at 445 nm (B). Arrows indicate laser flash. Numbers to the left of the curves indicate incubation time in hours.

When the ba_3-type oxidase was incubated with CO for several hours, fast components appeared in the CO recombination curve (Fig. 3A). The same observation was made in the case of the bovine heart aa_3-type oxidase (Fig. 3B). One can see that the contribution of the single monophasic component decreases upon incubation whereas fast components (τ values of about 70–80 μs and 0.5–0.6 ms) arise and increase during the 24 h incubation.

It was shown that the *bo*-type oxidase can reassociate with CO at a rate of about one order of magnitude higher in *E. coli* cells grown under copper-limited conditions [17]. Therefore, a reasonable explanation for the observed increase in rates of CO recombination might be the loss of copper during the prolonged incubation under experimental conditions. As one can see in Table 1, all three studied enzymes of ba_3, caa_3 and aa_3 types lost copper during 17–24 h incubation. Omitting CO from the samples followed by air bubbling and by laser flash in the open cuvette did not restore the initial enzyme properties, indicating that the observed changes in CO reassociation kinetics were irreversible. It should be mentioned that kinetic and copper measurement did not depend on whether or not the samples were illuminated by laser light during the experimental period.

4. Discussion

The method of laser flash photolysis of CO-oxidase complexes used by our group in previous studies [3,4,19] allows two groups of terminal oxidases to be identified. As we suggested previously such a classification is based on dependence of the time constant of CO reassociation on the presence of copper in the binuclear center of the enzyme [5]. This suggestion is supported by studies on oxidases of all known types: aa_3 from bovine heart, caa_3 from *B. halodurans* FTU, ba_3 from *P. denitrificans*, bo_3 from *E. coli* and *M. flagellatum* KT, *bd* from *E. coli*, and *bb* from *B. halodurans* FTU and putatively *bb* from *M. flagellatum* KT (unpublished data). The experimental study of Basu et al. [7] seems to support the result of our work. The exception is the copper-containing ba_3-type oxidase from *T. thermophilus* described earlier [7]. It was shown to recombine with CO on a fast timescale like the *bd*-type oxidase from *E. coli*. However, the study was conducted at only a single wavelength (440 nm) using whole cells. At this wavelength, the oxidases of the *bb* and *bd* types similar to their *B. halodurans* FTU and *E. coli* homologues [3] might contribute to the kinetics observed.

Fig. 1 in the present study shows that the *P. denitrificans* ba_3-type oxidase recombines with CO slightly more slowly than the aa_3-type oxidase from bovine heart. Our data are confirmed by theoretical calculations performed for oxidases of the ba_3 type from *T. thermophilus* and the aa_3 type from bovine heart [8]. According to our data, the CO recombination rate does not depend on species used, provided that they belong to one and the same superfamily. For example, oxidases of the caa_3 type from *B. halodurans* FTU [18] and of the ba_3 type from *P. denitrificans* [6] were shown to belong to the superfamily of heme-copper terminal oxidases like the classi-

Table 1
Metal content and copper to iron ratio in the studied bacterial and bovine heart oxidases

Enzyme and source of isolation	Content (mol/mol of enzyme)		Cu/Fe ratio
	Cu	Fe	
Paracoccus denitrificans ba_3-type oxidase			
upon 17 h incubation	0.21 ± 0.04	2.03 ± 0.05	0.21:2
before incubation	n.d.	n.d.	1.4:2
Bacillus halodurans FTU caa_3-type oxidase			
upon 24 h incubation	1.32 ± 0.01	2.99 ± 0.03	1.33:3
before incubation	2.80 ± 0.03	3.11 ± 0.06	2.7:3
Bovine heart aa_3-type oxidase			
upon 24 h incubation	1.85 ± 0.01	2.19 ± 0.04	1.72:2
before incubation	3.40 ± 0.01	2.07 ± 0.07	3.29:2

n.d., not determined.

cal objects of studies – oxidases of the aa_3 type from bovine heart and the bo_3 type from *E. coli* [2]. All of them show CO recombination time constants of the same order of magnitude. The aa_3-type oxidase from bovine heart possessing the heme-copper binuclear center recombines with CO only slightly faster than all bacterial oxidases of this superfamily but much more slowly than the *bd*-type and the *bb*-type oxidases containing no copper [5,23]. According to Shapleigh et al., a CO molecule might bind initially to Cu_B in the binuclear center and then migrate to the high-spin heme [24]. One might interpret the faster reassociation rates for the mitochondrial oxidase in terms of a closer apposition of both metal centers. Interestingly, our data are in good correlation with the high resolution three-dimensional X-ray structure of metal sites of *P. denitrificans* and of beef heart aa_3-type oxidases. It was shown that the heme a_3 iron to copper (Cu_B) interatomic distance is 5.2 Å in bacterial [25] and 4.5 Å in mammalian [26] enzyme.

As was found by our group, the increasing rate of CO recombination of the copper-containing oxidases upon incubation for several hours results in a loss of copper and the appearance of fast CO recombination kinetics typical of the non-copper oxidases (the *bb*-type oxidase of *B. halodurans* FTU and the *bd*-type oxidase of *E. coli*) (see Table 1 and Fig. 2). It should be mentioned that in all our previous experiments freshly isolated enzymes were used and the experiment usually took 1–2 h [3,4]. The fast components with a τ lower than 10 ms in experiments on copper-containing oxidases have never been seen by our group. Fast kinetics observed after long incubation of copper-containing oxidases resemble data on the *E. coli bo*-type oxidase obtained by Brown and coworkers [16]. In this paper, an acceleration of CO reassociation with membranes enriched in the *bo*-type oxidase as well as with the purified *bo*-type enzyme was found when the *E. coli* cells were grown under copper-limited conditions. In such a case, a fraction of the enzyme was suggested to lack Cu_B. Unfortunately, direct copper determinations have not been carried out. The suggestion of this group was supported by experiments on the *E. coli bo*-type oxidase mutated in putative copper ligands [16]: replacement of His^{333} or His^{334} with Leu were shown to accelerate, and of His^{284} with Leu to slow down the CO recombination rate compared with the wild type enzyme.

According to our results, the acceleration of CO reassociation with oxidases of the aa_3 type and the ba_3 type during long-term incubation closely correlates with the loss of copper, the iron content being constant in the enzymes. Interestingly, the ba_3-type oxidase contains a single copper ion and its loss leads to this kinetic acceleration. One may assume that a similar process takes place in the aa_3-type oxidase, where out of the three copper atoms, Cu_B is lost predominantly.

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